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SHORT COMMUNICATION

Breeding System and Seed Production in Pin-monomorphic Populations of a Distylous Perennial, *Ophiorrhiza japonica* (Rubiaceae) on Iriomote Island, Japan

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The breeding system and seed production of a distylous perennial, *Ophiorrhiza japonica*, were analyzed on Iriomote Island, located near the southwestern end of the Ryukyu Islands. On Iriomote, *O. japonica* is monomorphic with a pin morph. Artificial pollination experiments confirmed self- and intramorph compatibility in the populations, but automatic self-pollination was not detected. Seed set in 12 sampling plots in two natural populations was constant and abundant, indicating high pollinator availability. Our results indicate that self compatibility can be assumed to have been important in the establishment of the monomorphic populations on Iriomote Island. The loss of distyly is not characterized by specialization in self-fertilization, but rather intramorph compatibility can be assumed to have been more important in maintenance of the pin-monomorphic population as a factor facilitating outbreeding

Keywords: breeding system, distyly, morph ratio, *Ophiorrhiza japonica*, seed production

Ophiorrhiza japonica Blume (Rubiaceae) is a perennial herb widely distributed northern Vietnam through southern China to Taiwan and southwestern Japan (Pitard 1922, Hatusima 1975), and occurs in the humid understory of natural laurel forests and sometimes at streamsides. It typically exhibits distyly, comprising individuals with long styles and short stamens (pin morph) and individuals with short styles and long stamens (thrum morph; Lo 1990, Liu & Yang 1998). In the Ryukyu Islands, however, the breeding system is heterogeneous, with monomorphic pin morph populations occurring with distylous ones (Naiki *et al*. 2001, Nakamura *et al*. 2007). This illustrates the morph ratio variation and the breakdown of distyly on these islands and is obviously distinct from the breakdown of distyly in *O. amamiana* (Hatus.) Koh Nakam., Denda, Kameshima & Yokoka (= *O. japonica* var. *amamiana* Hatus.), which is associated with the evolution of homostylous, self-fertile flowers and tetraploidy (Nakamura *et al*. 2007). Aside from preliminary descriptions and counts of the number individuals of each morph, little is known of the factors leading to morph-ratio variation or the establishment of monomorphic populations.

Distyly has been reported in 26 families of angiosperms (Naiki 2012), and is generally considered to be a genetically controlled mechanism to promote efficient pollen transfer (Barrett 2002). Owing to intramorph incompatibility and negative frequency-dependent selection, distylous populations are expected to be composed of an equal ratio of two morphs (isoplethy) at equi-

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librium (Barrett & Shore 2008). Deviation from isoplethy has sometimes been observed (e.g. Thomson *et al.* 1998), however, and in extreme cases, results in the loss of one or the other morph (Martin 1967, Thompson *et al.* 1998). Deviations can be induced by stochastic processes, such as colonization or population fragmentation, and/or deterministic factors including the inter-morph difference in fitness components or ecological preferences and the alternation of compatibility status (Kéry *et al.* 2003). In particular, the loss of a morph is observed in distylous taxa with the collapse of intramorph incompatibility (Consolaro *et al.* 2011, Zhou *et al.* 2012), and in some cases, it is accompanied by specialization in self-fertilization (Sakai & Wright 2008) or the modification of reproductive components (Nicholls 1985). In this regard, assessment of the breeding system and its relationship to seed production is important for understanding factors that shape morph-ratio variation, including the breakdown of distyly.

In this study, the breeding system and seed production of *Ophiorrhiza japonica* was examined on Iriomote Island, an island located near the southwestern end of the Ryukyu Islands (Fig. 1). On Iriomote, despite the relatively large population size, populations of *O. japonica* are monomorphic with pin morph (Naiki *et al.* 2001, Nakamura *et al.* 2007). The loss of thrum morphs cannot be explained by recent demographic fluctuations. We therefore aimed to confirm compatibility in pin morphs and its relationship to reproductive output, which is an initial step toward obtaining a full understanding of the factors shaping the morph-ratio evolution of *O. japonica* on the Ryukyu Islands.

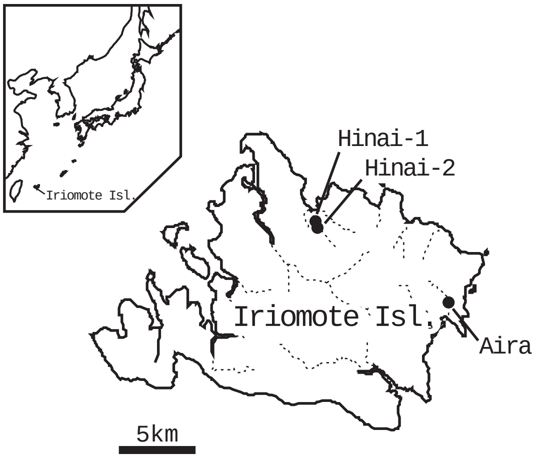

FIG. 1. Geographical locations of three study populations of *Ophiorrhiza japonica*.

Materials and Methods

Study species

Ophiorrhiza japonica is a rhizomatous forest herb typically occurring at streamsides on Iriomote Island. Individual plants produce one or more shoots 15–50 cm tall, have opposite ovate-lanceolate leaves, terminal inflorescences that bear 7 to 20 white salverform flowers. On Iriomote, *O. japonica* flowers from late January into February. Each flower lasts 3 or 4 days and is pollinated by insects such as hawkmoths and butterflies (Nakagawa pers. obs.). The fruit is boat-shaped and contains 100 to 300 seeds, each ca. 0.2mm in diameter The fruit is adapted to dispersal of the seeds by raindrops, so-called “splash-cup dispersal” (Nakanishi 2002). *O. japonica* in the Ryukyu Islands is diploid, with $2n = 2x = 22$ (Nakamura *et al.* 2003).

Study populations

Field research was conducted on three populations on Iriomote Island (Fig. 1): Hinai-1 (24°22′48″N, 123°49′21″E, 30 m a.s.l.), Hinai-2 (24°22′40″N, 123°49′19″E, 120 m a.s.l.) and Aira (24°20′27″N, 123°54′37″E, 25 m a.s.l.). Hinai-1 and Hinai-2 are located along Hinai River. Hinai-1 is about 500 m downstream from Hinai Falls. Hinai-2 is above the falls. In Hinai-1, *Ophiorrhiza japonica* occurs mainly along the riverbank where it forms large clumps vegetatively. In contrast, Hinai-2 comprises relatively small individuals occurring only on streamside rocks. In Aira, along Aira River, small clumps of *O. japonica* are sparse on streamside rocks or on the adjacent rocky riverbank. Vegetative spread is variable, but less notable than in the Hinai-1 population. Crossing experiments were performed on the Hinai-1 and Hinai-2 populations; seed production was measured for the Hinai-1 and Aira populations.

Crossing experiments

For the Hinai-1 and Hinai-2 populations, hand pollination experiments were conducted from 28 January to 14 February, 2001. Several randomly selected inflorescences were covered with fine (ca. 0.2 mm) nylon-mesh bags before anthesis, and open flowers and flower buds not subjected to the experiments were removed. Five treatments were assigned randomly to the inflorescences (Table 1):

(1) Open pollination: the bags were removed from the flowers and they were open-pollinated.
(2) Outcrossing: the flowers were emasculated before anthesis and artificially pollinated with pollen from other individuals.
(3) Self-pollination: open flowers were artificially self-pollinated.
(4) Bagging: the bags were left on the flowers with no experimental treatment until the stigma wilted.
(5) Stigma removal: stigmas were removed by cutting the tip of the buds.

To determine the percentage of seed set, developing fruit were harvested about one month after the treatments and preserved in 70% (v/v) EtOH. At harvest time, the fruit had developed to more than 5 mm in diameter. The fruit was dissected under a stereoscope. Developing seeds with a robust seed coat and wilted undeveloped ovules were clearly distinguishable. The number of each was scored, and the percentage seed set was estimated as the ratio of the number of developing seeds to the total number of ovules (i.e., the sum of developing seeds and wilted ovules). Differences in mean seed set among the treatments were tested by one-way ANOVA and by multiple comparisons with Tukey's HSD test separately for the two populations. Prior to the tests, the seed-set ratio was arcsine-transformed to meet the assumption of a normal distribution of variables (Sokal & Rohlf 1995). Statistical tests were performed with R var. 2.15.2 (R Core Team 2012).

Measurements of seed production

For Hinai-1 and Aira, sampling plots for the measurement of natural seed set were established according to the local distribution of *Ophiorrhiza japonica* (Table 2). In Hinai-1, because of the high shoot density due to vegetative growth, relatively small plots (3–5.25 m^2; Table 2) were sparsely placed within 50 m of the mountain trail, which were five or more meters away from the inflorescences used for the crossing experiments. In Aira, seven sampling plots between 1.5 and 11.25 m^2 were established according to the local patch size. In both populations, the plots were more than 5 m away from one another. The inflo-

TABLE 1. Mean seed set resulting from artificial pollination experiment in Hiani-1 and Hiani-2 of *Ophiorrhiza japonica*.

Population/ Treatment	No. of Flowers (No. of Inflorescences)	Seed set (%)*
Hinai-1		
Open pollination	30 (11)	56.9 (4.2)^a
Outcrossing	25 (10)	60.7 (4.6)^a
Self-pollination	23 (11)	58.2 (4.2)^a
Bagging	24 (9)	1.3 (0.6)^b
Stigma removal	22 (10)	0.3 (0.2)^b
Hinai-2		
Open pollination	25 (16)	63.8 (4.9)^a
Outcrossing	25 (11)	71.8 (3.6)^a
Self-pollination	25 (14)	72.9 (3.5)^a
Bagging	5 (5)	0.6 (0.4)^b
Stigma removal	17 (10)	0.0 (0.0)^b

*, values with the same letter are not significantly different ($P>0.05$) by Tukey's HSD tests followed by one-way ANOVA performed separately for the populations; the standard errors are in parentheses.

rescences within each plot were counted to obtain floral density. In addition, four isolated flowering individuals 5–23 m downstream from the nearest Aira plots were also sampled to determine seed set under conditions of low floral density.

Inflorescences with developing fruit were collected from the 12 plots in two populations and from the four isolated individuals in Aira (Table 2). The harvested inflorescences were preserved in 70% (v/v) EtOH. Measurement of seed set was determined for two to five (average 3.12) developing fruits from each inflorescence (Table 2). Estimation of the seed set ratio was performed according to the procedure in the crossing experiments. To test the significance of differences in mean seed set among the plots, the arcsine-transformed seed set ratio was subjected to nested ANOVA (Zar 1999), with plots nested within the populations. The results from scoring seed set in the isolated individuals, ignoring their sampling irregularity, were pooled and nested with the measurements obtained from Aira. Statistical computations were conducted using R 2.15.2 (R Core Team 2012).

Results and Discussion

Despite the limited number of flowers in bagging treatment at Hinai-2, the mean seed set in the crossing experiments showed clear differences among the five treatments and were consistent between the two populations (Table 1). Seed set of about 60% or higher was observed in open pollination, outcrossing and self-pollination treatments. In contrast, seed set in bagging and stigma removal treatments was nearly zero. One-way ANOVA showed significant differences in the seed set ratio of the treatments in Hinai-1 (F = 126.00, P < 0.001) and Hinai-2 (F = 95.51, P < 0.001), and multiple comparison with Tukey's HSD tests confirmed significant differences between the two groups of treatments (Table 1).

The results revealed several characteristics of the breeding system of *Ophiorrhiza japonica* on Iriomote Island. First, the almost complete absence of seed set after removal of the stigmas indicates that seeds are the result of sexual reproduction. Second, substantial seed production

TABLE 2. Natural seed set at the 12 sampling plots of two study populations (Hinai-1 and Aira) of *Ophiorrhiza japonica*.

Population/ plot	Area (m^2)	Floral Density (No./m^2)	Distance (m)*	No. of Fruits (No. of Inflorescences)	No. of Ovules per Fruits Mean ± SE	Seed set (%) Mean ± SE	Percentage of fruits with seed set more than 10%
Hinai-1							
H1	5.25	11.8	-	15 (5)	207.5 ±15.7	59.4 ± 6.5	86.7
H2	5.00	17.8	-	15 (5)	249.3 ± 4.2	73.0 ± 4.6	100.0
H3	3.00	16.0	-	15 (5)	260.9 ± 9.3	58.6 ± 4.8	93.3
H4	3.00	12.0	7.0	15 (5)	254.7 ± 12.1	53.9 ± 8.7	80.0
H5	3.00	18.7	-	15 (5)	276.6 ± 9.2	70.7 ± 6.4	100.0
Aira							
A1	12.00	3.4	-	17 (5)	196.2 ± 5.1	74.6 ± 5.5	100.0
A2	9.00	4.3	-	15 (4)	258.4 ± 11.2	74.7 ± 3.0	100.0
A3	14.00	4.6	-	14 (5)	220.1 ± 7.3	71.8 ± 7.6	100.0
A4	11.25	1.2	6.0	14 (4)	226.6 ± 11.1	69.7 ± 5.7	100.0
A5	4.50	15.3	-	15 (5)	236.5 ± 5.5	82.2 ± 4.8	100.0
A6	1.50	8.0	-	12 (3)	182.6 ± 7.5	76.4 ± 8.5	91.7
A7	1.50	9.3	6.0	13 (5)	238.2 ± 8.8	55.5 ± 5.7	100.0
Isolated individuals in Aira**							
1	-	-	22.0	6 (1)	292.0 ± 9.2	74.3 ± 8.1	100.0
2	-	-	7.5	6 (2)	238.5 ± 8.0	25.3 ± 8.5	83.3
3	-	-	7.5	5 (1)	192.0 ± 9.3	55.7 ± 22.8	60.0
4	-	-	6.0	5 (1)	177.2 ± 10.5	88.2 ± 3.5	100.0

*, distance to the nearest flowering individual located outside the plot. "-" indicates that the plot was set as a part of a continuous distribution. **, area and floral density were not defined, and are denoted with "-".

with artificial pollination showed a lack of intramorph compatibility. Third, self-compatibility was evident because seed set in outcrossing and self-pollinating treatments did not differ significantly in both populations. Fourth, assuming self-compatibility, the almost complete lack of successful seed production in bagging treatment means that automated self-pollination did not occur, and that pollination services are critical for seed set. Finally, mean seed set in open pollination treatments was not significantly different from outcrossing treatments, indicating that the populations are free from pollinator limitation.

A total of 198 developing fruits from 12 sampling plots in two populations (Hinai-1 and Aira) and four isolated individuals in Aira (Iso1–4) were analyzed to determine natural seed set (Table 2). Among the 12 plots, mean seed set ranged from 53.9% (H4) to 82.2% (A5), and averaged 60.7%. Despite a wide range in floral density and the setting of the plots, differences in seed set were not significant among the populations (F = 1.934, P = 0.192) nor in the plots nested within populations (F = 1.757, P = 0.064). In addition, 80% or more fruit showed more than 10% seed set (Table 2). Because the crossing experiments revealed the necessity of pollination services for seed set, the abundant and constant seed production in the plots indicated high levels of pollinator availability in the two populations. In particular, one of the isolated individuals in Aira, which was separated by 22 m from the nearest flowering individual, showed 74.3% seed set (Isolated Individual 1 in Table 2). Although seed production was examined in only one season, these results support the lack of pollinator limitation in the Iriomote populations of *Ophiorrhiza japonica*.

Ophiorrhiza japonica on Iriomote Island comprises only pin-morph individuals (Naiki *et al*. 2001, Nakamura *et al*. 2007). Our results show that *O. japonica* on Iriomote island is self- and intramorph compatible (Table 1). This allows the pin-morph individuals to maintain their populations without the presence of thrum morphs, and could be a primary factor leading to a breakdown of distyly. Despite being self-compatible, automatic self-pollination was not detected (Table 1). Self-compatibility is therefore not directly connected to the assurance of reproductive output. While some distylous taxa showed dependence on self-fertilization for seed production in pin-monomorphic populations (Sakai & Wright 2008, Consolaro *et al*. 2011), the breakdown of distyly in *O. japonica* is not characterized by specialization in self-fertilization. Instead, seed production was constantly high, presumably due to high pollinator availability (Table 2). Although the contribution of pollinator-mediated self- or geitonogamous pollination to seed production was not evaluated, the pin-monomorphism in *O. japonica* may be maintained by a greater incidence of outbreeding, which may be due to sufficient pollinator services and intramorph compatibility.

In the Ryukyu Islands, pin-monomorphic populations of *Ophiorrhiza japonica* occur widely (Naiki *et al*. 2001, Nakamura *et al*. 2007). The self- and intramorph compatibility in pin morphs found in this study can explain such a morph-ratio pattern as a deterministic factor. It has been reported that populations of *O. japonica* in the Ryukyu Islands were established as a result of major vicariance-dispersal events (Nakamura *et al*. 2010). Self-compatibility of pin-morph plants may facilitate their persistence through demographic fluctuations, possibly leading to pin-biased morph ratio within a population. This explanation is only hypothetical, since little is known of reproductive behavior of thrum morphs. Further comparative studies of morphs across a wider geographical range are needed to clarify the underlying causes responsible for the morph-ratio in *O. japonica* in the Ryukyu Islands.

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